The biochemistry of blood coagulation

THE BIOCHEMISTRY OF BLOOD COAGULATION

H.C. Hemker

INTRODUCTION

Thrombin is the central enzyme in hemostasis and thrombosis. The study of its generation has been prompted by the many diseases in which hemostasis and thrombosis play a role. The bleeding disorders like hemophilia A are obvious but rare examples. Much more common is the other extreme: the excessive and unknown reaction of the hemostatic process known as thrombosis and its sequel embolism. Thrombosis on basis of atherosclerosis and thrombosis in the veins are very important pathological processes. Evidence is rapidly accumulating that not only (micro)thrombosis is an important complication of atherosclerosis but also plays a role, maybe even the main role, in the genesis of atherosclerosis. Thrombosis in one form or another may therefore be considered to be a key event in well over half of all deaths in the western society, including well-known diseases as coronary infarction, stroke, circulation disturbances in the legs, kidney disease etc. Thrombin generation therefore is a suitable subject of research in a biochemistry department of a medical faculty. On the other hand it shows so many novel features not recognized in enzymology until now, that it cannot fail to interest even the most basicly interested biochemist.

Since the middle of the last century, thrombin is known to clot blood, to cause a fibrinogen solution to turn into a jelly-like mass. This phenomenon is most impressing in vitro, but probably is of minor importance in hemostasis and thrombosis. Since the early sixties, it has been recognized that thrombocytes (blood platelets) play the major role in these processes. For some time this tended to obscure the role of thrombin. More recently, however, it became increasingly apparent that it is precisely the effect of thrombin on platelets that causes platelets to engage in thrombosis and hemostasis. Thrombin is not the only effector for these cells. Prostaglandins, ADP, serotonin etc. all play their role. Thrombin, however, is the agent that makes the platelet reactions irreversible and recent research in our laboratory has shown that it is involved much more early in the platelet reactions than has been supposed before. Anyhow, the view that clotting of blood is observed

in venous thrombi only and that therefore thrombin plays a role in venous thrombosis only, although still widely supported among practitioners and pathologists, is no longer tenable.

WHAT IS THROMBIN?

Thrombin is a proteolytic enzyme of the serine protease family. It is not unlike trypsin, in that it is specific for bonds next to arginin. Its main or B-chain containing the active serine shows extensive homologies with trypsin, chymotrypsin and elastase as well as with the other proteases of blood coagulation that we will discuss presently. The B-chain contains aminoacids (in bovine material) and is S-S linked to the A-chain, 58 residues in length. The primary structure of both chains has been solved in the laboratory of Magnusson.

The function of the A-chain is as yet obscure. It can be safely guessed that it will play a role in the striking specificity of this enzyme. This specificity is already illustrated by its action on fibrinogen. The large fibrinogen molecule (M.W. 360.000) contains scores of potential vulnerable bonds next to its many arginins. Yet only two of these are split by thrombin. This generates the fibrin monomer that spontaneously polymerizes and thus causes 'clotting' per se. Thrombin has a multitude of other functions in hemostasis. It activates the fibrin stabilizing factor (factor XIII). This factor in its activated form links lysin residues to glutamic acid residues in adjacent units of the polymer and thus covalently links the clot. It also activates procoagulant factors (factors V and VIII) and this is coupled in a positive feedback system to its own generation. The way in which these factors act will be discussed below.

Thrombin also partakes in a negative feedback because it splits its proenzyme (prothrombin = factor II) into a product that is less readily activated than intact prothrombin. Thirdly, and perhaps most important, thrombin acts on thrombocytes. It causes them to aggregate a basic reaction of these cells in plugging a hole in a blood vessel as well as the first recognizable event in thrombosis and probably in the generation of atherosclerosis. It also causes them to make available the phospholipids that are necessary for the clotting process: another form of positive feedback. Thrombin, of course, is as dangerous as it is useful. When it is present in the blood stream even in tiny amounts it causes a serious clinical picture known as disseminated intravascular clotting, that often is the final lethal complication in such diverse diseases as solutio placentae, traumatic brain damage or leukemia. One is not surprised to find a very efficient scavenger system for thrombin in the plasma. The main component is antithrombin 3. It quickly combines with thrombin (and other activated clotting factors) to form a stoichiometric pro-

duct with no enzymatic activity. An important recent event in blood coagulation research is the development of chromogenic substrates. These are tri- or tetrapeptides linked to a p-nitroanilide residue. They can be designed to be relatively specific for thrombin or other clotting esterases and thus make it feasible to estimate the enzymes by spectrophotometric assay. This circumvents much technical problems adherent to the study of the coagulation system by measuring clotting times.

WHAT IS PROTHROMBIN? HOW IS IT ACTIVATED? ROLE OF VITAMIN K

Prothrombin is the zymogen for thrombin. It is a single chain molecule of amino acid residues. The molecular weight is 72,000. It contains both the A- and the B-chain of thrombin but these only account for slightly more than half of the molecule.

In other words, there is an unusually large activation peptide. In order to generate the two chain trombin molecule it has to be split in two sites. The primary structure of prothrombin is known as well as the probable order of bond splitting during activation. In the activation peptide there is a site vulnerable to the action of thrombin. This splits the activation peptide in two parts, called fragment 1 and fragment 2. These regions have different and specific functions in the activation process. Fragment 2 can bind to factor V. Fragment 1 binds to phospholipid. Binding to factor V and phospholipid of the prothrombin molecule greatly enhances its liability of being activated as we will see later. Of particular interest is the way in which fragment 1, the N-terminal end of prothrombin, binds to phospholipid. For this function it is necessary that ten glutamic acid residues in this part of the molecule are converted into γ-carboxyglutamic acids. γ-carboxyglutamic acid (gla) residues are formed by carboxylation of glutamic acid residues in the polypeptide that but for these carboxylations is identical to prothrombin. This carboxylation is an unusual reaction in that it requires reduced vitamin K, O_2 and CO_2 but no biotin or ATP. Recently, the liver enzyme responsible for this reaction has been isolated in our laboratory. If by a lack of vitamin K or by the administration of vitamin K antagonists (the so-called oral anticoagulants, mostly coumarin derivatives) the carboxylation step cannot be carried out, no or at least not enough prothrombin is synthesized and the blood level drops. The precursor, i.e. the uncarboxylated prothrombin precursor either remains in the liver cell (as in the rat and chicken) or does not reach the blood stream (as in men and cows). This circulating descarboxyprothrombin also known as Protein Induced by Vitamin K Absence, similar to factor II (PIVKA-II), may slowly generate thrombin and also in other ways disturb coagulation tests in anticoagulated patients. In fact, it was by these

effects that it was first recognized. It is one of the factors that makes the control of oral anticoagulant treatment a difficult task and thus contributes to the occasional failure of this useful therapy. Apart from this, PIVKA-II can be isolated from plasma and because it is completely identical to prothrombin but for the capability to bind to phospholipids, it is a perfect model to study the lipid binding of this clotting factor. It may be mentioned already now that the other vitamin K dependent clotting factors (i.e. factors VII, IX and X) also show gla residues, that also serve to bind them to phospholipid surfaces. When no vitamin K is present (c.q. when its action is blocked) analogs of these factors, called PIVKA-VII, PIVKA-IX and PIVKA-X, occur in the blood stream.

The bonds that need to be split in prothrombin in order to liberate thrombin are clipped by proteolytic enzymes. This can be done by trypsin, by certain snake venoms or by activated factor X. These reactions occur in free solution but are not very efficient. Under physiological circumstances a more sophisticated biochemical mechanism is operative.

PROTHROMBINASE

The normal physiological activator of prothrombin consists of factor X_a bound to a phospholipid surface next to a molecule of factor V_a. Factor X_a is the activated form of factor X, one of the vitamin K dependent coagulation factors. This factor occurs in the blood in a two-chain form. The heavy chain (MW 44,000) contains the active serine and is S-S bound to the light chain (MW 17,000) that contains 12 gla residues in its N-terminal part. Upon activation, the mechanism of glycopeptides is removed from the N-terminal part of the heavy chain. Unlike thrombin, this activated enzyme in this case does possess a gla containing region, and it is via this region that it can bind to phospholipid. Extensive comparisons carried out in our lab between factor X and PIVKA-X, the decarboxy form of the enzyme found in vitamin K deficiency, show that PIVKA-X can be activated like factor X and then acquires properties indistinguishable from factor X_a when reactions are studied that take place in free solution. When the reaction takes place at an interface, the properties are very different indeed.

Factor V_a is a 100,000 MW two-chain protein that arises from a 360,000 MW single-chain precursor in an as yet not completely clear way. The activation under physiological circumstances is carried out by thrombin.

It should be mentioned that no enzymatic activity of factor V_a is known. Activation here does not mean a proenzyme-enzyme conversion, as it does with all other coagulation factors except factor VIII. Early experiments showed that factor X_a alone is capable of splitting prothrombin but that its catalytic action is enhanced up to

several thousand fold by the addition of phospholipids and factor V_a that both are inactive as such.

Early kinetic experiments showed that prothrombinase is formed by independent reversible adsorption of the factors V_a and X_a on the phospholipid surface.

It thus appears that an interesting question in general enzymology arises. Why is it that an enzyme (factor X_a) is much more potent when it acts in concerted action with an accessory factor, also called paraenzyme (factor V_a) adsorbed at an interface? The question may be of much more general importance than for the activation of prothrombin alone. Not only does a similar situation occur in the activation of factor X, but also the same configuration is encountered several times in the complement system. It also can be recognized in oxidative phosphorylation and it might indeed be a feature of many reactions occurring at membrane-solution interfaces.

THE REACTIONS LEADING TO THE ACTIVATION OF FACTOR X

The factor X activated enzyme is the vitamin K dependent, hence gla-containing, hence lipid binding factor IX_a. Precisely like factor X_a it needs for its full activity adsorption onto a phospholipid surface and a para-enzyme, in this case factor VIII. Factor VIII remains a somewhat enigmatic protein and I will not discuss it to any extent here. It is sufficient to say that it probably is a polymer of two different proteins, one of which is active in coagulation. Like factor V it is activated by thrombin.

Factor IX_a in its turn arises from factor IX in a reaction catalyzed by factor XI_a. The mechanism of formation of factor XI_a has been unraveled in the last few years. It is a direct consequence of contact of blood with negatively charged wettable surfaces.

In the presence of such a surface, factor XII adsorbs from the plasma and in adsorbing undergoes a conformational change that makes it a suitable substrate for kallikrein. Kallikrein, however, does not act as such. In plasma it is bound to high molecular weight kininogen (HMWK). Via HMWK that contains a domain of about 120 residues, 30% of which are histidines, and that hence bears a strongly positive charge, kallikrein binds to the negative surface, next to factor XII and is capable of activating factor XII. On the other hand, factor XII_a can activate prekallikrein when it adsorbs, also via HMWK.

This mutual activation leaves us with the question of how the process starts. If both factor XII and prekallikrein were absolutely inactive pro-enzymes, the reaction could not be triggered without the help of another enzyme. At the moment we think that the proenzymes are not completely inactive, that some interaction is always possible but that this is easily repressed by the antiprotease abundant in plasma.

By adsorption to a negative surface two phenomena occur

simultaneously:
a. factor XII becomes a better substrate
b. the concentration of the reactants near the surface is enhanced.

This the factors engage in a strongly non-linear interaction that breaks through the protective action of the antiproteases.

Factor XII_a activates factor XI that like (pre)kallikrein is bound to the negatively charged surface by HMWK. Factor XI_a then activates factor IX. The pathway here described is called the intrinsic pathway because no material alien to the blood plays a role in it.

There is another pathway. A lipoprotein present in almost all cells and called tissue thromboplastin can interact with a vitamin K dependent plasma protein factor VII to form a complex in all probability completely analogous to the prothrombin and intrinsic factor X activating complexes that can activate factor X. Factor X_a in its turn can activate factor VII and between these two proenzymes a situation of reciprocal activation may exist not unlike the situation between factor XII and factor XI described above. Only here the reaction occurs at a phospholipid solvable interface.

It becomes more and more clear that the factor VII dependent pathway is not completely independent of the intrinsic pathway. It has been known for a long time and has been again found with modern methods that factor VII acts upon factor IX. In fact under conditions that only a limited amount of tissue thromboplastin is present, the action of factor VII upon factor X is markedly enhanced by the presence of the factors VIII and IX, but independent of the contact factors.

Factors VIII and IX thus seem to act as an accessory factor X_a activator that can be triggered by factor VII and tissue thromboplastin.

THE FUNCTION OF COMPLEX ENZYMES

The data in the literature only state that phospholipids and factor V_a, each alone enhance the activity of factor X_a as a prothrombin activator and that, when both are present together their action is markedly increased. We tried to find out what the mechanism behind this phenomenon is.

To this end we purified factors X_a, V_a and II, and we synthesized phospholipids of known composition. Then we estimated the kinetic parameters of the conversion of factor II by the different possible activating enzymes, i.e. factor X_a, factor X_a - phospholipid, factor X_a - factor V_a, factor X_a - factor V_a - phospholipid. The rate of formation of thrombin at different prothrombin concentrations was measured by subsampling from the incubation mixture into a cuvette containing S 2238, a chromogenic substrate for thrombin and assessing the amount of thrombin from the rate of color production observed. In this way Line-

weaver-Burk plots of each of the enzymes could be obtained. It was seen that - but for a rare exception to be discussed later - straight Lineweaver-Burk plots were obtained.

We found that phospholipids decrease the K_m by about 1000-fold and that factor V increases V_{max} by about the same factor.

It is obvious that the overall rate enhancement attained by the combination of these effects can be enormous. The concentration of prothrombin in plasma is about 2 μM and it is seen that addition of phospholipid moves the K_m from above this value to well below it. Experiments on the factor X activating complex showed that completely comparable kinetics were observed here with factor X in the role of substrate and factors IX_a and VIII acting as enzyme and paraenzyme. The effects of the phospholipids and factor V are clearly different and the next task would be to seek a mechanistic explanation for the observed facts.

THE PHOSPHOLIPIDS

Already in 1967, we had found that excess phospholipid inhibits the formation of prothrombinase and we explained this phenomenon by assuming that the reaction of prothrombinase with prothrombin takes place in the two-dimensional compartment formed by the phospholipid-water interface. Addition of an excess of phospholipid increases this interface and dilutes the protein components in it. If this is the right explanation then the kinetic constants observed in bulk solution are only apparent constants and should be recalculated so as to represent the situation at the surface. For this we set out to determine the apparent K_m and V_{max} at a range of phospholipid concentrations.

It appeared that K_m shows a clear variation with the phospholipid concentration. From the literature the dissociation constant and the number of binding sites of prothrombin for the type of phospholipid we used could be obtained. With the aid of this constant the concentration of prothrombin at the surface could be calculated. It turned out that at every phospholipid concentration the variable apparent K_m calculated on basis of the bulk phase concentration corresponded to one fixed concentration of adsorbed prothrombin. The same phenomenon could be still more elegantly demonstrated with the factor X converting enzyme. Here we found a method to estimate the dissociation constant and the number of sites for factor X onto the phospholipid on the same day and in the same preparations as used for determination of the kinetic constants. This method is based on the fact that a proteolytic protein from the venom of Russells Viper (called in short RVV-X) activates factor X in free solution but does not act on the adsorbed enzyme. From the velocity of factor X formation

in a mixture of phospholipid and factor X, the amount of free factor X thus be immediately assessed. When this is done in a series of concentrations a Scatchard plot can be constructed that yields both the dissociation constant and the number of binding sites for factor X on phospholipid.

Also in this case it became clear that the reaction shows its half maximal reaction velocity at a fixed number of substrate molecules adsorbed per amount of lipid. Apart from the substrate also the enzyme has to adsorb on the phospholipid. Under the conditions of our experiments with prothrombinase we saw that V_{max} increased slightly with the concentration of phospholipid. As V_{max} is proportioned to the enzyme concentration this was thought to reflect a more complete binding of the enzyme at higher phospholipid concentrations. The fact that this phenomenon is less pronounced than the effect on substrate concentration can be explained from the higher affinity of the enzyme (factor X_a) for the surface and from the fact that only small amounts of factor X_a are present i.e. the amount of lipid present is in excess over the amount of factor X_a and therefore the binding of factor X_a is favored.

These results all point in the direction that the reaction takes place between the adsorbed enzyme and the adsorbed substrate. The interface seems to form a 'two-dimensional reaction vessel' in which the molecules meet. Why should the reaction be favored by this arrangement. On first sight it is clear that the concentration at the surface is higher but a second thought learns that this as such will not increase the interaction velocity. For the molecules to interact they have to meet and it is not clear that adsorbed molecules are free to meet. Two arguments can be brought forward that favor the model proposed. The first is a theoretical one. From various types of experiments an estimate can be made of the lateral mobility of proteins adsorbed onto phospholipids. One can calculate the collision frequency and compare it to that in free solution. It turns out that the collision frequency of adsorbed protein may be easily some 100 to 1000 times higher than that in the free solution. The second argument in fact shows that an experimental change in diffusion coefficient does increase the reaction rate. We could show that the procoagulant activity of synthetic phospholipids sharply increases at the transition temperature of the phospholipids. As it can be assumed that the lateral diffusion coefficient increases sharply above the transition temperature, this can be accepted as evidence in favor of our model. Apart from increased collision frequency adsorption at an interface can act favorably by two other mechanisms. In free solution colliding molecules will make first contact at random places of their surfaces. When adsorbed they are oriented because one specific part of the molecules - the gla containing

N-terminal end - interacts with the phospholipids. Colliding adsorbed molecules therefore will make first contact at restricted places on their surface and the molecules may be so constructed that this causes more collisions to result in interaction.

Thirdly, the adsorption may cause conformational changes in the proteins and thus make them more reactive. This possibility is as difficult to refute as it is to prove and is only interesting when other explanations fail. In the contact activation mechanism it may be the only remaining possibility. It is as yet not absolutely necessary to postulate it in this case. We concluded that adsorption onto phospholipid increases the number of favorable collisions between the molecules, therefore acts as if increasing the concentration of substrate and thus causes a decrease of K_m.

THE PARAENZYMES

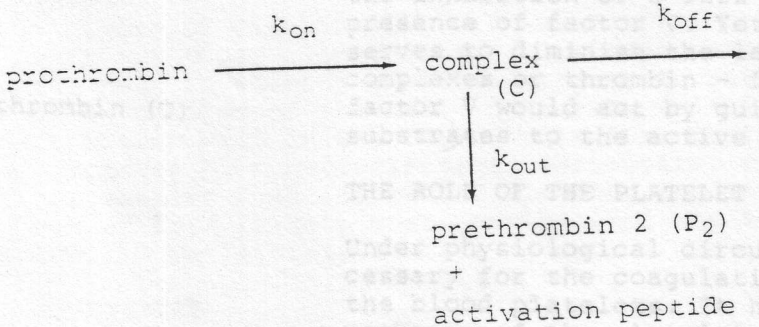
The activation of factor X involves the splitting of one peptide bond only. The k_{cat} of this reaction is increased by factor VIII, the accessory protein in this case. We therefore have to consider an influence of this protein on the reaction mechanism of the splitting.

Special attention should be paid to the possibility of the occurrence of noncovalent complexes. The increase of any forward rate constant in this mechanism will result in an increase of the observed k_{cat}. With small amide substrates acylation is the rate-limiting step. The rates that are observed with small substrates are explained by a k_{cat} of 10-100. Without factor VIII the k_{cat} of factor X activation is much lower (10^{-4}). With factor VIII k_{cat} is restored to about the level seen with small substrates (~2). This suggests that rather than accelerating the catalytic mechanism per se, factor VIII brings about conditions that make the enzyme as effective with large substrates as with small ones. This suggests that the mechanism of bond breaking as such is not involved, because this is not different in large and in small substrates. Rather would one expect an effect on interactions that are known to be different for these two types of substrates, i.e. the noncovalent interactions. We will see that in the case of prothrombin more arguments can be brought forward for this view.

The case of prothrombin is more complicated though. Here two peptide bonds have to be split in the substrate. The minimal possible reaction scheme therefore is:

It appears from an analysis of the reaction products obtained with different forms of prothrombinase that in the absence of factor V, P_2 + F is the main reaction product, whereas in its presence thrombin is formed directly.

So in the absence of factor V, P_2 dissociates readily from the enzyme but in its presence not. In order to discuss this phenomenon we will employ a simplified form of the reaction scheme:



Factor V can prevent the production of P_2 in one of two ways. Either k_{out} is decreased or the steady state concentration of C is lowered by an increase of k_{off} relative to k_{on}. We cannot exclude that k_{out} is decreased by factor V. Actually binding of the activation peptide to factor V has been observed and it is also known that there is a relatively light noncovalent binding between F and P_2. It can be easily argued, however, that a decrease of k_{out} can not be the only mechanism involved. In the first place one would like to have a unifying explanation for the action of the paraenzymes in both complexes studied, but a decrease of k_{cat} can never explain the effect of factor VIII on factor X activation as only one bond splitting is involved there. In the second place k_{cat} of thrombin formation in the presence of factor V is about 50 s^{-1}. This means that both k_{on} and k_{out} should be at least 50 s^{-1}. The rate of P_2 formation in the absence of factor V was estimated by us to be 0.5 s^{-1} and the k_{cat} of thrombin formation was 0.05 s^{-1} under these circumstances. This is incompatible with forward rate constants of 50 s^{-1} or more. So one or both of the kinetic constants k_{on} and k_{off} have to be increased by factor V.

An interesting observation is that although the action of factor X_a on its natural substrate prothrombin is inhibited by the presence of a competing small substrate (S 2238), the action of factor X_a on the small substrate is not inhibited by the presence of prothrombin. Even when prothrombin is present at concentrations of about K_m so that one would expect that about 50% inhibitor would occur. In our opinion this can only be explained if prothrombin - factor X_a complexes occur that have the active center of factor X_a available for the splitting of a small

substrate. If this is true, then factor V would act by diminishing the amount of that type of complexes. Unfortunately, technical difficulties prevent to investigate the inhibition of S 2222 conversion by prothrombin in the presence of factor V. Yet we may postulate that factor V serves to diminish the level of prothrombin - factor X_a complexes or thrombin - factor X_a complexes. That is, factor V would act by guiding the large molecular weight substrates to the active center of the enzymes.

THE ROLE OF THE PLATELET IN COAGULATION

Under physiological circumstances the phospholipids necessary for the coagulation reactions are provided by the blood platelets. It has been shown that the plasma membrane of the platelet is asymmetrical and that the phospholipid composition of the outside has no significant procoagulant properties whereas those at the inside have. Especially phosphatidyl serine (PS) is essential in providing the negative charge that is a prerequisite for binding of the K dependent clotting factors. This phospholipid is almost entirely found at the inner surface of the membranes. It is wellknown that platelets that are triggered for their hemostatic function show a release reaction. As a part of this phenomenon factor V_a is transferred from storage vesicles inside the platelet to the outside. We could show that if platelets are triggered by thrombin and collagen, procoagulant phospholipids appear at the outside of the plasma membrane. This phenomenon is not accompanied by platelet breakdown as can be judged from the fact that no platelet intracellular enzymes are released. On the other hand the material stored in the platelet granules (serotonin, factor V) is released. The procoagulant phospholipids remain an integral part of the platelet and, for more than 90% can be spun down with the platelet. We hypothesize that parts of the plasma membrane of the platelet turn inside out (flip-flop) so as to present their procoagulant inner surface when they are triggered. At the same time factor V_a becomes available from the inside of the platelets. This explains the presence of highly specific factor X_a binding sites at the surface of activated platelets. The factor X_a at the surface of a platelet is immune to activation by antithrombin 3. The platelet reaction therefore provides a rich source of prothrombinase activity. Also because platelets active in hemostasis stick to each other and to subendothelial structures, they create a sponge-like structure in the crevices of which flow will not wash away the thrombin formed. Thrombin again will induce release and flip-flop in neighboring platelets. The actions of blood platelets and the blood coagulation factors thus mutually reinforce each other.

CONCLUSION

From the biochemical point of view the blood coagulation mechanism offers the possibility of studying reactions and does cover patterns that are not easily found elsewhere.

Years of careful clinical observation have helped to sort out those illnesses that, as experiments of nature, can help in solving these questions. On the other hand, investigation of the basic phenomena underlying thrombin formation will in the end help in interfering with the processes of atherosclerosis, thrombosis, embolism, intravascular coagulation and bleeding tendencies, that together form largely more than half of the total death toll in our society at this moment.

REFERENCES

In view of the general nature of this overview further studies of the literature are best carried out on the hand of specialized review articles.

1. Hemker, H.C.: Capita Selecta: Vorming en functies van thrombine. Ned. Tijdschr. v. Geneesk., 126:198,1982.
2. Miletich, J.P., Majerus, P.W.: Properties of the factor Xa binding site on human platelets. J.biol.Chem. 253:6908, 1978.
3. Suttie, J.W., Jackson, C.M.: Prothrombin structure activation and biosynthesis. Physiol. Rev. 57:1. 1977.
4. Zwaal, R.F.A.: Biochim.biophys.Acta 515:163, 1978.
5. Zwaal, R.F.A., Hemker, H.C.: Blood cell membranes and haemostasis. Haemostasis, 11:12,1982.